Metaphase Arrest with Centromere Separation in *polo* Mutants of *Drosophila*

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Abstract. The *Drosophila* gene *polo* encodes a conserved protein kinase known to be required to organize spindle poles and for cytokinesis. Here we report two strongly hypomorphic mutations of *polo* that arrest cells of the larval brain at a point in metaphase when the majority of sister kinetochores have separated by between 20–50% of the total spindle length in intact cells. In contrast, analysis of sister chromatid separation in squashed preparations of cells indicates that some 83% of sisters remain attached. This suggests the separation seen in intact cells requires the tension produced by a functional spindle. The point of arrest corresponds to the spindle integrity checkpoint; Bub1 protein and the 3F3/2 epitope are present on the separated kinetochores and the arrest is suppressed by a *bub1* mutation. The mutant mitotic spindles are anastral and have assembled upon centrosomes that are associated with Centrosomin and the abnormal spindle protein (Asp), but neither with γ-tubulin nor CP190. We discuss roles for Polo kinase in recruiting centrosomal proteins and in regulating progression through the metaphase–anaphase checkpoint.

Key words: mitosis • spindle checkpoint • *Drosophila* • Polo kinase • cell cycle

Introduction

The Polo-like protein kinases (Plks)[1] are a conserved family of enzymes that play a variety of roles in the passage of cells through M phase (for reviews, see Glover et al., 1998; Nigg, 1998). They are named after the *Drosophila polo* gene originally identified through a recessive maternal effect lethal mutation. Flies homozygous for the original *polo*^{1} allele can develop to adulthood due to the weakly hypomorphic nature of this mutation and the provision of wild-type protein from their heterozygous mothers. However, they do exhibit spindle pole defects at several developmental stages including multiply branched spindles in syncytial *polo*^{1}-derived embryos, and spindles with broad poles and a low frequency of circular mitotic figures in larval neuroblasts (Sunkel and Glover, 1988; Llamazares et al., 1991). Requirements for *polo* during male meiosis are also evident from the chromosome nondisjunction and failures in cytokinesis seen during meiosis of *polo*^{1} testes (Sunkel and Glover, 1988; Carmena et al., 1998; Herrmann et al., 1998). A more recent examination of meiosis in *polo*^{1} eggs and at the onset of their zygotic development indicates abnormalities in microtubule organizing centres in the meiotic spindle, the sperm aster, and the astral arrays of microtubules associated with the polar bodies (Riparbelli et al., 2000).

These roles of Polo kinase are echoed in other organisms, suggesting conserved function. Disruption of the fission yeast counterpart, *plo1*, leads to formation of monopolar spindles (Ohkura et al., 1995). Interfering with enzyme activity using antibodies can also lead to the formation of monopolar spindles in *Xenopus*, or human cells (Lane and Nigg, 1996; Qian et al., 1998). In human cells, this appeared to be linked to a failure of the centrosome to increase its microtubule nucleating activity upon mitotic entry. Indeed, a direct role for *polo* in mitotic entry is suggested by the finding that *Xenopus* Polo-like kinase, Plx1, copurifies with and can activate cdc25, and may thus play a role in the positive feedback loop that operates during p34^{cdc2} activation at the G2-M transition (Kumagai and Dunphy, 1996; Abrieu et al., 1998; Qian et al., 1998).

A requirement for the Plks to promote the onset of cytokinesis also appears to have been conserved from the yeasts to the metazoans. Disruption of the fission yeast *plo1* leads also to the formation of multinucleate cells in which neither an actin ring nor a septum has been formed. Overexpression of *plo1*$^{+}$ in fission yeast, on the other

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[1]*Abbreviations used in this paper:* APC, anaphase-promoting complex; Asp, abnormal spindle protein; γ-TuRC, γ-tubulin ring complex; plk, Polo-like protein kinase.

hand, leads to the formation of multiple septa at any stage of the cell cycle, indicating the potential of the enzyme to overcome the dependence of this process upon the completion of mitosis (Ohkura et al., 1995). Expression of an activated form of mammalian Plk in budding yeast has also been found to drive the formation of multiple septa, suggesting this is a conserved property of the enzyme (Lee and Erikson, 1997). In *Drosophila*, the requirement of *polo* for cytokinesis appears to be linked to the need to reorganize the central region of the spindle in late M phase (Herrmann et al., 1998). In budding yeast, Cdc5p is also shown to play a role in regulating cytokinesis, and the polo-box, a conserved motif in the noncatalytic domain of Plks, is required for this (Song and Lee, 2001).

There is also evidence that the polo-like kinases can activate certain functions of the anaphase-promoting complex (APC), an E3 ubiquitin-protein ligase that directs the degradation of anaphase inhibitors, Pds1p in budding yeast (Cohen-Fix et al., 1996) and Cut2p in fission yeast (Funabiki et al., 1996), and the mitotic cyclins (for reviews, see Townsley and Ruderman, 1998; Yanagida, 1998). Descombes and Nigg (1998) showed that the addition of catalytically inactive polo-like kinase to colony-stimulating factor (CSF)-arrested extracts of *Xenopus* eggs blocks the Ca^{2+}-triggered destruction of cyclin B and inactivation of $p34^{cdc2}$ and also prevented the destruction of exogenous APC-dependent substrates. Furthermore, M phase exit would not take place in this system after immunodepletion of Plx1, but could be restored by the addition of catalytically active enzyme. The mouse polo-like kinase will also phosphorylate and activate the bacterially expressed APC components Cdc16 and Cdc27 in vitro (Kotani et al., 1998). The mitotic cyclin Clb2p is not degraded in mutants for the budding yeast plk, Cdc5p (Shirayama et al., 1998), whereas overexpression of Cdc5p increased APC activity and decreased the levels of Clb2p, an outcome not obtained with a "kinase-dead" mutant (Charles et al., 1998).

Until now, a requirement for *polo* in regulating APC in *Drosophila* has not been apparent from the phenotypes of the alleles that have been studied. $polo^1$ mutants, for example, are able to progress through development, as a function of the weakly hypomorphic protein is sufficiently supplemented by maternally provided wild-type protein. Consequently, it has been difficult to assess fully the functions of Polo kinase in $polo^1$ somatic cells which are capable of progression through multiple cell cycles. In this paper, we describe the mitotic phenotype of two strongly hypomorphic alleles, $polo^9$ and $polo^{10}$, which block the proliferation of diploid tissues. Cells of these mutants appear poised to initiate anaphase and sister kinetochores are pulled apart. Chromatids undergoing partial separation can remain attached through their telomeres and do not migrate to the spindle poles. We discuss potential roles for Polo kinase in regulating specific functions of checkpoint proteins and the APC at the metaphase–anaphase transition.

Materials and Methods

Immunoblotting

Extracts of larval brains were prepared for immunoblotting according to Scaerou et al. (1999). 6 brains were used per lane for wild-type, 7 for $polo^1$, and 12 per lane for $polo^{9/10}$. This ensured equal protein loading, which was tested by probing with the C4 mouse monoclonal antibody to actin. The Polo and γ-tubulin proteins were identified by probing with MA81 rabbit anti-Polo and GTU88 mouse anti-γ-tubulin antibodies.

Giemsa-stained Preparations of the Larval Central Nervous System

Third-instar larvae were washed and dissected in PBS. The associated imaginal discs were removed and the brains were incubated in 45% acetic acid for 30 s, then in 60% acetic acid for 3 min. The brains were then squashed hard between a poly-lysine–coated slide (Sigma-Aldrich) and a siliconized coverslip. The slides were frozen in liquid nitrogen and the coverslip flipped off with a scalpel blade. The preparations were then dehydrated in 75 and 100% ethanol for 5 min each before being left to air dry. The preparations were then stained in Giemsa and mounted in DPX. Preparations were observed at 60 and 100× objective on a compound microscope. In experiments where colchicine was used, the dissected brains were incubated with 10^{-5} M colchicine, in Schneiders tissue culture medium (Trueman and Bate, 1988) containing FCS, for 30 min, 1 h, 2 h, or 4 h, before being incubated in 45% acetic acid.

Immunostaining

Immunostaining of whole mount preparations of third-instar larval brains was carried out by first dissecting the larvae in PBS and then fixing in 3.7% formaldehyde for 20 min. Brains were washed in PBS, then permeabilized in 0.3% Tween 20 in PBS for 10 min. The tissue was "blocked" by a 40-min incubation in PBS containing 10% FCS before being incubated overnight with the primary antibodies in PBS containing 0.1% Tween 20 (PBST) containing 2.5 μg/ml RNase, at 4°C. Brains were then washed in PBST and incubated with a 10^{-2} dilution of secondary antibodies in PBST containing 10% FCS for between 2 and 4 h. Brains were finally washed in PBST before being incubated in propidium iodide to stain DNA. The preparation was mounted in Vectashield.

Immunostaining with the 3F3/2 antibody was performed according to Bousbaa et al. (1997), except that no taxol was used, and the staining of α-tubulin carried out simultaneously by adding YL1/2 into the primary antibody incubation, and the appropriate suitable secondary antibody into the subsequent incubation.

Microscopy was performed using a Nikon Optiphot compound microscope with a Bio-Rad Laboratories MRC 1024 Scanning Confocal attachment. Images were captured using Lasersharp software and manipulated in Adobe Photoshop.

Results

Strong polo Hypomorphs Show Mitotic Arrest in Larval Brain Cells

We wished to isolate strong hypomorphic alleles of *polo* in order to examine their effect upon mitosis in somatic cells. As P-elements generally insert either into the 5′ regulatory regions or coding sequences to downregulate or inactivate their target genes, we sought to identify such alleles in a collection of mutants generated by P-element–mediated mutagenesis (Deak et al., 1997). We discovered two new *polo* alleles, $polo^9$ and $polo^{10}$, that have their lethal phase during late third larval instar development and have respective mitotic indices that are elevated about 11- and 8-fold above cells of wild-type larval brains (Fig. 1 and Table I). This is considerably higher than $polo^1$ that has a mitotic index about fourfold greater than wild-type in this tissue. $polo^1$ mutants produce comparable levels of protein to wild-type, but the kinase is thought to be defective due to a failure of an activating phosphorylation (Tavares et al., 1996). In contrast, the amounts of Polo kinase in the $polo^9$ and $polo^{10}$ mutants are barely detectable (Fig. 2 B). This is a consequence of the insertion of P-elements in the two lines into the noncoding regions of the first exon of

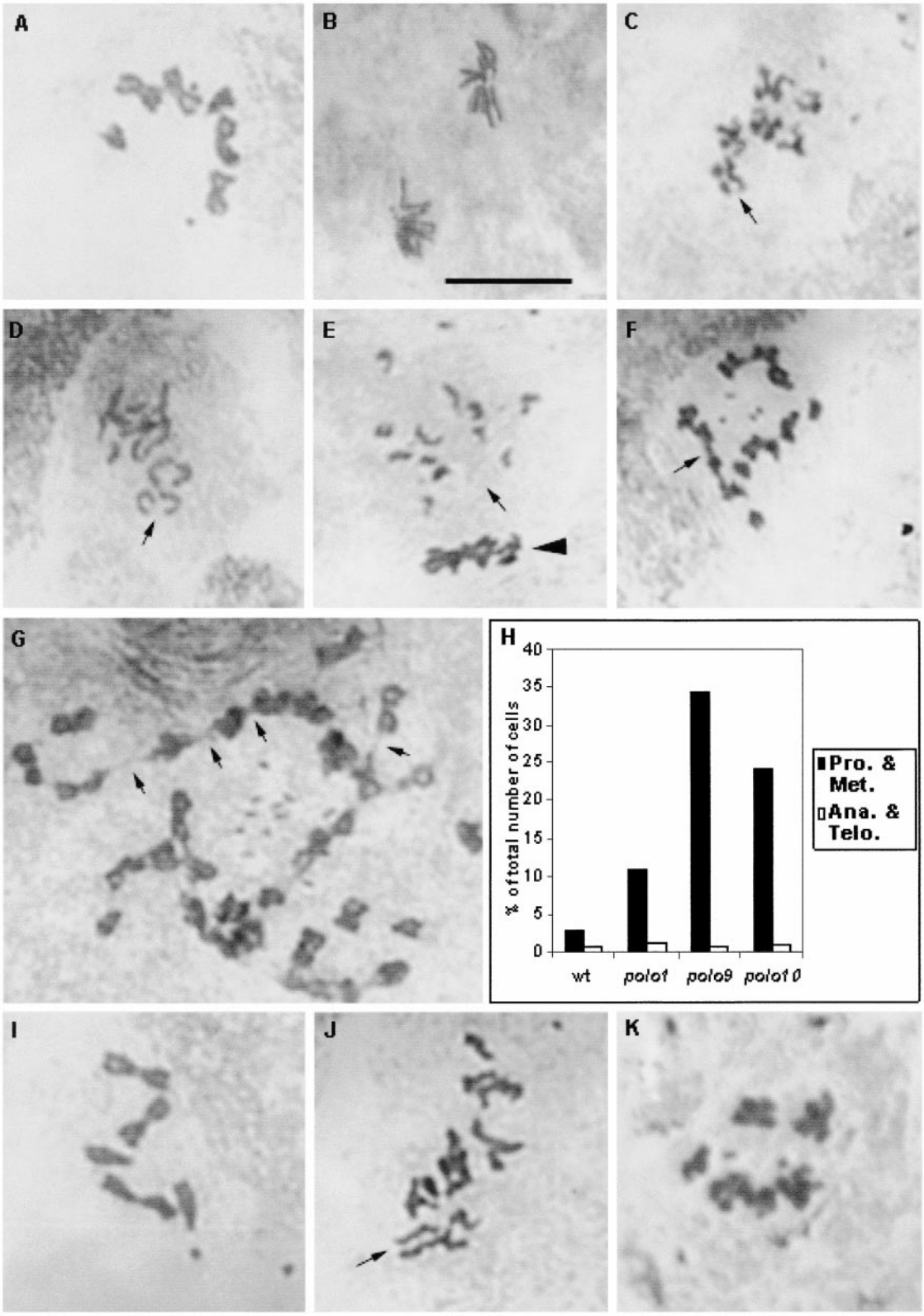

Figure 1. Mitotic figures from wild-type, *polo*^{9}, and *polo*^{10} brains. (A) A wild-type metaphase figure. (B) A wild-type anaphase figure. (C) Hypercondensed mitotic chromosomes from *polo*^{9}. The arrow points to a pair of separated sister chromatids. (D) *polo*^{10} cell showing separated sisters (arrow) that could be at early anaphase. (E) An anaphase (arrow) and metaphase (arrowhead) figure from *polo*^{10}. (F) A tetraploid *polo*^{10} cell in which several sister chromatids appear separated throughout their length, and yet joined at their telomeres (arrow). (G) A polyploid *polo*^{9} cell in which chromosomes form long chains attached at their telomeres (arrows). (H) Bar graph showing the proportion of cells at prophase and metaphase (Pro. & Met.) or anaphase and telophase (Ana. & Tel.) in wild-type (wt), *polo*^{1}, *polo*^{9}, and *polo*^{10} brains. (I) Wild-type cell from a larval brain treated for 4 h with colchicine. (J) *polo*^{10} cell after 30 min colchicine treatment. The arrow marks separated sisters. (K) *polo*^{10} cell after 2 h colchicine treatment.

Table I. Analysis of the Mitotic Phenotype of polo Alleles in the Larval Central Nervous System

	Cells scored*	Prophase and metaphase‡	Circular mitotic figures	Anaphase and telophase	Mitotic cells: aneuploid or polyploid§	Mitotic index‖	M/A¶
	n	%	%	%	%		
Wild-type	2,521	2.8	0	0.5	1.2	3.3	5.8
polo^{1}	1,793	10.4	0.7	1.0	2.3	11.9	11.6
polo^{9}	1,616	32.9	1.4	0.7	16.1	35.6	51.4
polo^{10}	1,723	22.9	1.2	1.0	11.3	25.0	26.1
polo^{10} *bub1*	1,766	3.1	0.5	0.4	49.2	3.7	8.3

In addition to the above quantitation, numbers of pairs of sister chromatids showing separation were also counted in *polo*^{10} cells in the presence and absence of colchicine. Of 272 chromatid pairs scored in untreated *polo*^{10} cells, 17.2% were separated, compared with 17.0% of 176 chromatid pairs scored in colchicine-treated preparations. 40% of untreated *polo*^{10} cells had at least one separated pair of sister chromatids. This figure was similar (36.6%) after colchicine treatment.

*Total number of cells scored in 10 fields (60× objective) from 5 brains for each genotype.

‡Cells were scored as being metaphase if chromosomes were closely associated and if they contain some sister chromatids that appeared to be still attached.

§Note, this is percentage of mitotic cells and not total cells.

‖The mitotic index is scored as the number of mitotic cells as a percentage of age of total cell number.

¶Metaphase/anaphase ratio.

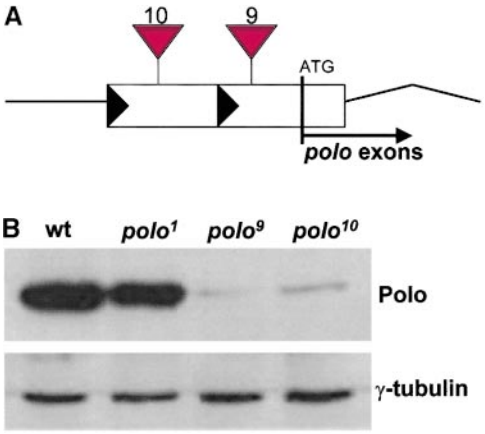

Figure 2. Sites of P-element insertions in *polo*^{9} and *polo*^{10} and relative expression levels of their gene products. (A) Schematic diagram of insertion sites in which P-elements are indicated by the filled red triangles above the linear map at positions −34 and −176 nucleotides upstream of the initiator ATG codon of the Polo protein. Two major starts for the initiation of transcription are indicated by the horizontal black triangles at positions −130 and −210 nucleotides. Exons are indicated by the open box and the first intron by the kinked line. (B) Western blots to compare the levels of Polo kinase and γ-tubulin in wild-type (wt), *polo*^{1}, *polo*^{9}, and *polo*^{10} larval brains (see Materials and Methods).

the *polo* gene at a position that affects either one (*polo*^{10}) or both (*polo*^{9}) of the two *polo* transcription units (Fig. 2 A). The levels of enzyme in *polo*^{9} are less than in *polo*^{10}, consistent with the sites of insertion, and they account for the relative severity of the mutant phenotype indicated by the mitotic indices.

Examination of mitotic chromosomes in squashed preparations of the central nervous systems from *polo*^{9} or *polo*^{10} larvae indicated several characteristic features. First, by comparison with wild-type mitotic cells (Fig. 1, A and B), chromosomes are much more highly condensed, indicating that the cells have been delayed in mitosis for some time (Fig. 1, C, E, and F). The majority of cells appear arrested in a metaphase-like state in that most sister chromatids (83%) are still joined at their centromeres, and the proportion of figures in which chromosomes are well separated at anaphase is relatively low, although in absolute numbers it is comparable to that in wild-type cells (Fig. 1 H and Table I). A proportion of cells are polyploid (Fig. 1, F and G, and Table I), suggesting that they have been able to escape the metaphase block and undergo a further cell cycle in the absence of chromosome segregation or cytokinesis. A low proportion of circular mitotic figures could be seen, as reported previously for *polo*[13]. In ~40% of cells, at least one set of the sister chromatids appear to have prematurely separated (arrows in Fig. 1, C and D; see also the legend to Table I). In addition, chromosomes frequently appeared to be connected through their telomeric regions. Fig. 1, F and G, show polyploid cells in which many chromosomes are linked in this way (arrows). This phenotype strongly resembles that described previously in the mutant *UbcD1* gene that encodes a class I ubiquitin-conjugating (E2) enzyme (Cenci et al., 1997). The mitotic index of the *polo*^{9} and *polo*^{10} brains did not change significantly after colchicine treatment, suggesting that the population of cells in this tissue could not respond further to disruption of spindle integrity (Fig. 1, J and K). A similar frequency of sister chromatid separation was observed independently of whether the preparation had been made in the presence or absence of colchicine.

polo Differentially Affects Centrosomal Components

Defects in spindle poles and other microtubule organizing centres have been reported previously for *polo*^{1}. However, the *polo*$^{9/10}$ mutants have allowed us to examine for the first time the effects of greatly diminished levels of the Polo kinase on the spindles and centrosomes of somatic cells. Examination of whole mount preparations of *polo*$^{9/10}$ larval brains stained with antibodies to α-tubulin to reveal the mitotic spindle confirms the results seen with Giemsa-stained preparations. It reveals a very high mitotic index with the majority of cells arrested in a metaphase-like state. The arrested cells all have bipolar spindles with robust arrays of spindle microtubules, but lacking asters at their poles (Figs. 3–6). Consistent with the previously observed failure of the CP190 antigen to assemble onto centrosomes in syncytial *polo*^{1}-derived embryos, CP190 does not concentrate in the centrosomes of *polo*$^{9/10}$ neuroblasts, but is scattered throughout the spindle with some tendency to accumulate around the condensed chromosomes (Fig. 3, K and L). γ-Tubulin is also missing from the spindle poles (Fig. 3, E and F). The protein cannot be seen in these particular micrographs, although other preparations show weak staining, suggesting it is dispersed throughout the mutant cells. This interpretation is supported by the detection of undiminished levels of γ-tubulin by Western blotting of extracts of these mutant brains (Fig. 2). In contrast, both centrosomin (Fig. 3, B and C) and abnormal spindle protein (Asp) (Fig. 3, H and I) are both present at the spindle poles in the mutant cells, indicating that Polo kinase function is not required for their localization.

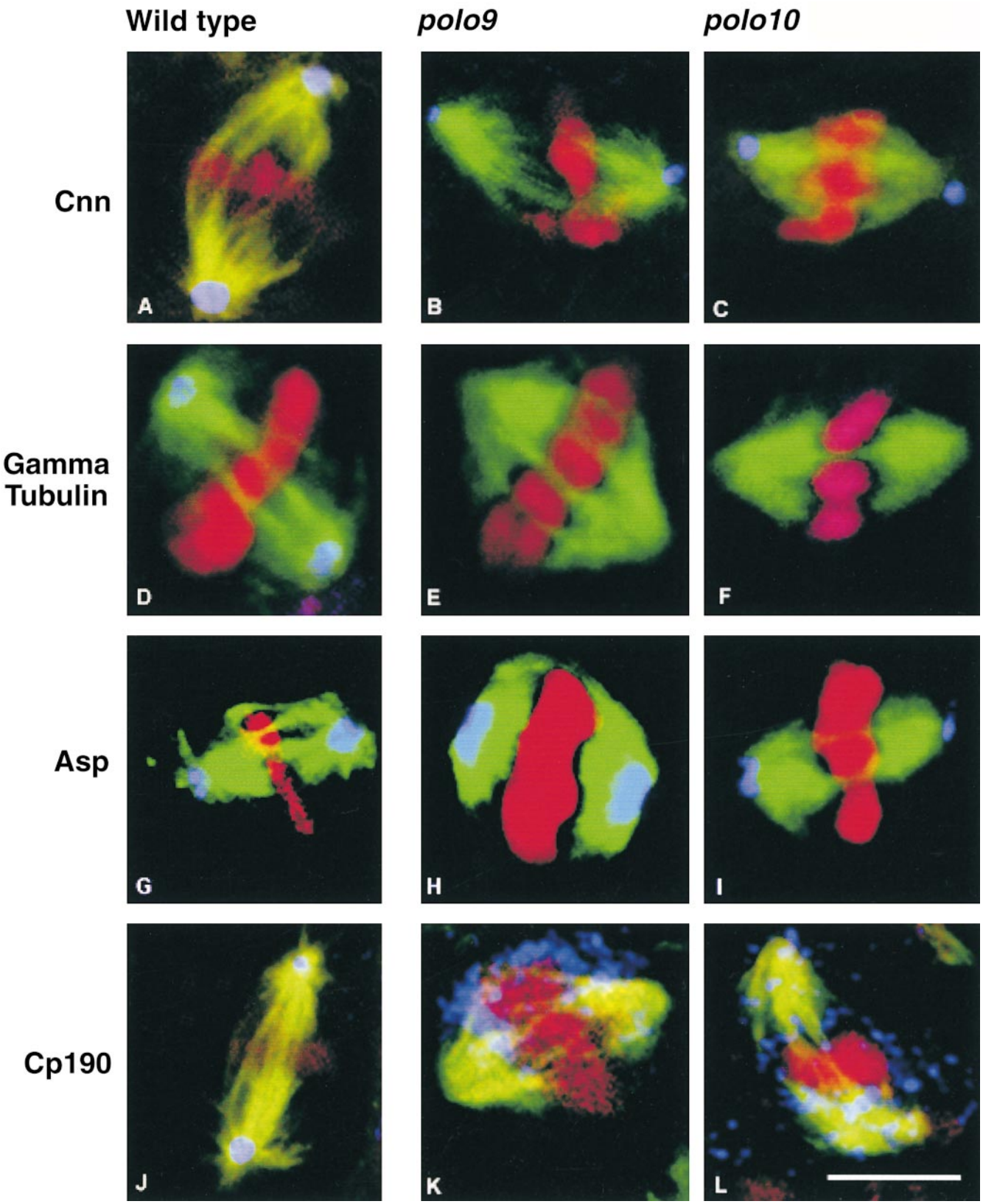

Figure 3. Distribution of centrosomal antigens in wild-type, *polo*^{9}, and *polo*^{10} cells. In all cases, wild-type cells are shown in the left panels, *polo*^{9} cells the middle panels, and *polo*^{10} cells the right panels. Spindle microtubules revealed by immunostaining with the YL1/2 antibody are shown in green, DNA stained with propidium iodide in red, and the centrosomal antigen in blue. (A–C) Centrosomin (Cnn) is revealed using a rabbit antibody from Heuer et al. (1995). (D–F) γ-Tubulin was detected using the mouse monoclonal antibody GTU88 (Sigma-Aldrich). (G–I) The Asp was detected using the rabbit antibody Rb3133 (Saunders et al., 1997). (J–L) CP190 was detected using the rabbit antibody RB188 (Whitfield et al., 1988). Bar, 5 μm.

Sister Chromatids Have Undergone Partial Separation in Strong polo Hypomorphs

It appeared from the above immunostaining experiments that the condensed chromatin had a broader distribution about the central part of the spindle in the *polo*^{9} or *polo*^{10} mutant cells than in wild-type cells at metaphase. We were curious to know whether this reflected any separation of the centromeric regions of the chromatids, so we localized the product of the gene *proliferation disrupter* (*prod*) in *polo*^{9} and *polo*^{10} cells. This protein localizes principally to the centromeric regions of chromosomes 2 and 3 (Torok et al., 1997). In wild-type cells at metaphase in which chromosomes are fully aligned on the metaphase plate, four punctate regions of Prod staining can be seen corresponding to the second and third chromosome pairs in which the sister centromeric regions are still tightly adjoined (Fig. 4 A). In *polo*^{9} (Fig. 4 B) or *polo*^{10} cells (Fig. 4 C), we frequently observed eight dots in two lines of four separated by 2–5 μm in the central region of the 10-μm long spindle. Approximately 80% of pairs of centromeric regions showed separation in these whole mount preparations of brains (see the legend to Fig. 4). This appearance suggests that movement of the centromeric regions of the chromosomes towards the spindle poles has been initiated and that the sister kinetochores are being pulled apart. The broad distribution of the chromosomes in these preparations contrasts with their highly condensed appearance in the squashed preparations. This may indicate that the chromatin is highly stretched when associated with the intact mitotic spindle. This broad distribution of chromatin in whole mount preparations is in contrast to compact condensed chromosomes in the metaphase arrest in *fizzy* (Fig. 4, D–F), a gene required to activate the anaphase promoting complex for degradation of both cyclin A and cyclin B (for a review, see Hershko, 1999). The distribution of Prod in *fizzy* cells indicates that the sister centromeres have not separated; only four dots of staining are seen and these are not aligned but randomly positioned within the area occupied by the condensed chromosomes.

polo^{9} *and polo*^{10} *Cells Bear the Signature of Checkpoint Arrest*

The segregation of sister chromatids into daughter cells at anaphase is normally prevented until a stable bipolar attachment is made between the kinetochores and the spindle microtubules. This process is monitored by the spindle integrity checkpoint which requires the function of a conserved complex of proteins originally identified in budding yeast as the products of the genes *BUB1*, *BUB3* (Hoyt et al., 1991), *MAD1*, *MAD2*, and *MAD3* (Li and Murray, 1991). In metazoan cells this complex localizes to unattached kinetochores to inhibit the activation of the APC (for a review, see Amon, 1999). Mutations in the *Drosophila* counterpart of one of these proteins, Bub1, indicate that it is required for the fidelity of chromosome segregation (Basu et al., 1999). The Bub1 protein associates with kinetochores as wild-type cells from the larval central nervous system progress to metaphase (Fig. 5 A), and is abruptly lost at anaphase (Fig. 5 B). We found metaphase-like arrested *polo*^{9} or *polo*^{10} cells in which 16 dots of Bub1 staining could be seen corresponding to the separated sister kinetochores (Fig. 5, C and D). Thus, the kinetochores appeared to have undergone alignment and the first stages of separation and yet Bub1 remained associated with them.

A phosphoepitope recognized by the 3F3/2 monoclonal antibody is also found at kinetochores that are reportedly not under tension (Gorbsky and Ricketts, 1993). It has been thought that when bipolar attachments are made, and tension at the kinetochore is established, the epitope becomes dephosphorylated and so it is no longer recognized by the 3F3/2 antibody. In *Drosophila* wild-type neuroblasts, the 3F3/2 epitope can be seen weakly at the kinetochores and at the spindle poles at metaphase (Fig. 5 E) and is lost from the chromosomes at anaphase (Fig. 5 F). Interestingly, spindle pole staining of 3F3/2 is not seen in the *polo*^{9} and *polo*^{10} mutants, probably reflecting the disruptions of the centrosome discussed above (Fig. 5, G and H). However, staining remains often as two separate lines of eight dots corresponding to the sister kinetochores of the four pairs of chromosomes (Fig. 5, G and H). Thus, this tension-sensing checkpoint epitope is present at kinetochores in the mutant cells even though one might expect the kinetochores to be under tension as they are pulled toward the spindle poles.

In the unperturbed mitotic cycle, cyclin A is abundant in prophase (Fig. 6 A) but is degraded ahead of cyclin B which is still abundant at metaphase (Fig. 6 D). This is accentuated during spindle integrity checkpoint arrest. After colchicine treatment, for example, cells arrest at a point at which cyclin A has undergone degradation allowing cyclin B to accumulate to higher than usual levels (Whitfield et al., 1990). In metaphase-arrested *polo*^{9} and *polo*^{10} neuroblasts, cyclin A has been degraded (Fig. 6, B and C), whereas cyclin B is present at elevated levels, particularly concentrated in the regions between chromosomes on the metaphase spindle (Fig. 6, E and F).

Thus, by three criteria both *polo*^{9} and *polo*^{10} cells appear to be delayed at the spindle integrity checkpoint, and yet they have initiated anaphase chromosome movements that have pulled apart the centromeric regions of the sister chromatids.

bub1 Releases polo^{10} *Cells from Checkpoint Arrest*

To determine whether indeed *polo*^{10} cells were in a state of checkpoint arrest, we made a double mutant construct with *bub1* and monitored mitotic progression in the larval central nervous system. The mitotic index in the double mutant was comparable to wild-type (Table I), and cells could be observed at all mitotic stages (Fig. 7, A–D). The spindles still showed the characteristic *polo* defects at their poles, which were anastral and showed no staining for CP190 (Fig. 7, E and F). CP190 antigen was present in the vicinity of the mitotic chromosomes (Fig. 7 E) and remained associated with telophase nuclei (Fig. 7 F). Stained squashed preparations were similar to those reported for the *bub1* mutant alone (Basu et al., 1999) and showed sister chromatids already separated at metaphase (Fig. 7 G), and lagging chromatids at anaphase (Fig. 7 H, arrow). This would account for the increased number of aneuploid cells that were observed (Table I). The double mutant also showed an elevated number of polyploid figures that could

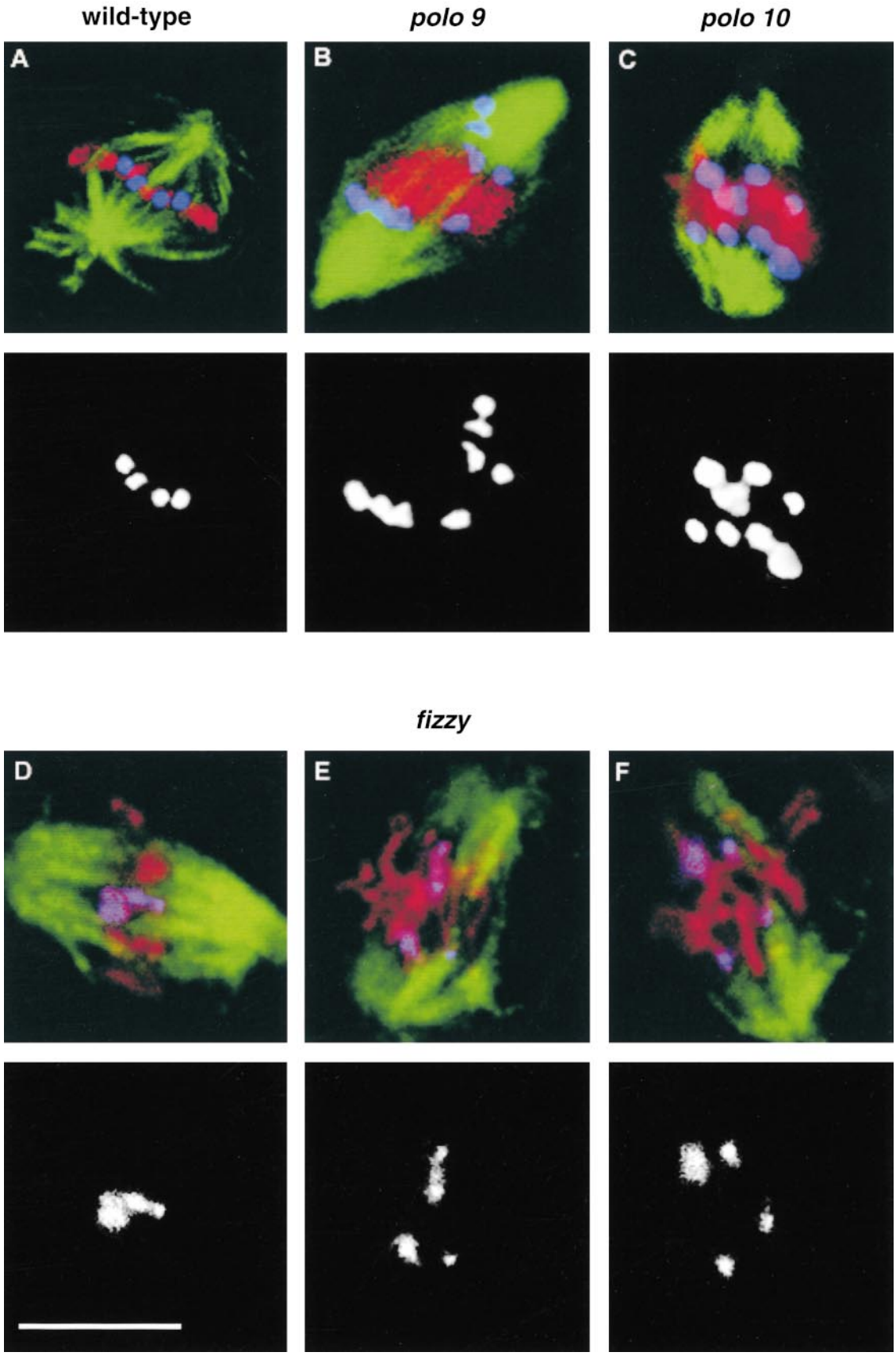

Figure 4. Localization of Prod in wild-type (A), *polo* (B and C), and *fizzy* (D–F) cells. Spindle microtubules stained with the rat monoclonal antibody YL1/2 are stained green. DNA is stained red. Prod (blue) was detected using a rabbit antibody (Torok et al., 1997). Merged images are shown in the top panels with the separated channel for Prod staining below. Bar, 5 μm. 79.3% of 213 clear pairs of sister centromeric regions were scored as having separated in immunostained *polo*^{9} cells. A similar frequency of separation (78.6% of 112 pairs) was observed in *polo*^{10} cells. Centromere separation was not observed by anti-Prod staining in the *fizzy*^{x4} mutant.

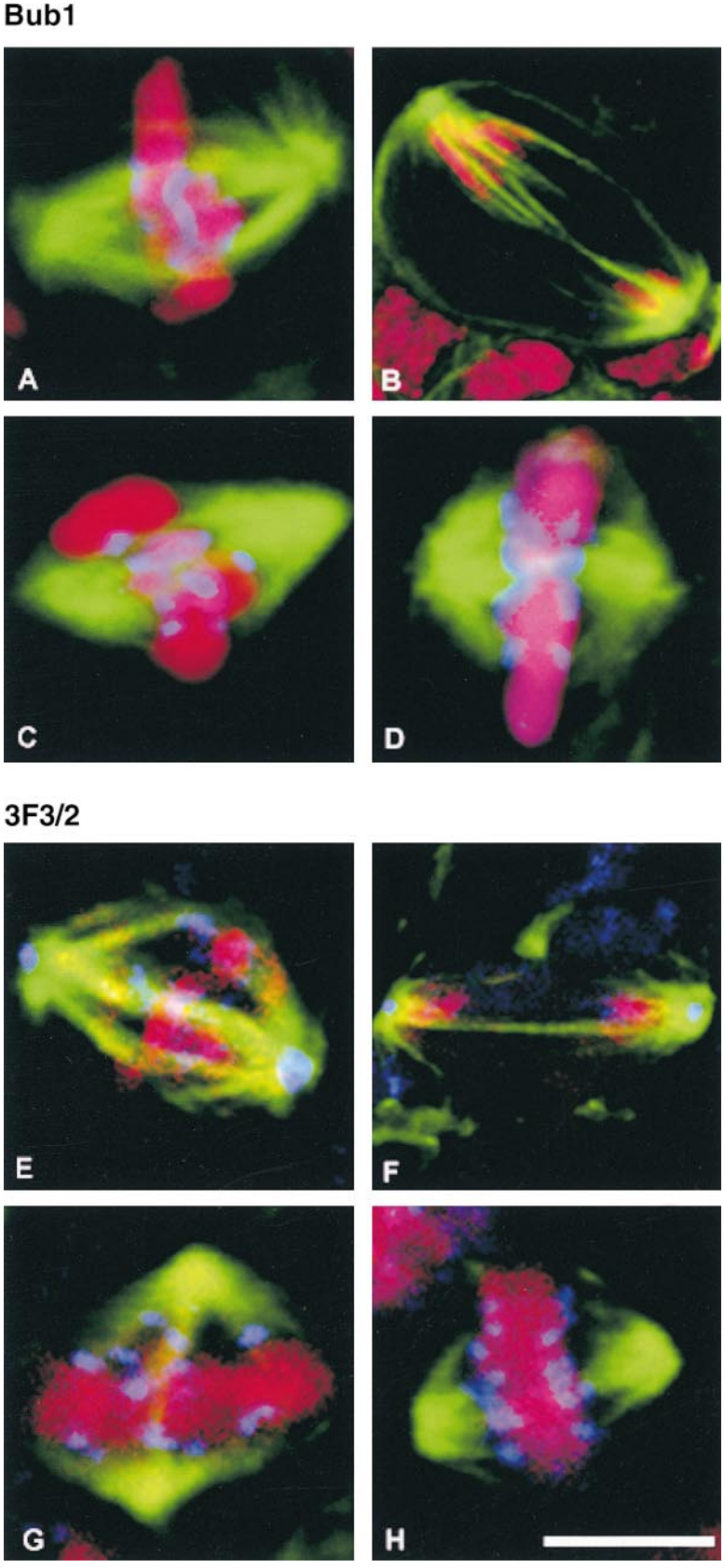

Figure 5. Association of Bub1 and the 3F3/2 epitope with the mitotic apparatus in wild-type and *polo* mutant cells. In all panels, spindle microtubules are stained green and DNA stained red. Bub1 (A–F) or 3F3/2 (G–L) staining are shown in blue. (A and B) Wild-type cells at metaphase and anaphase, respectively. (C and D) *polo*^{9} cells showing Bub1 staining with the rabbit antibody Rb666 (gift of C. Sunkel, University of Porto, Porto, Portugal) on the separated kinetochores. (E and F) Wild-type cells at metaphase and anaphase, respectively, stained with the 3F3/2 mouse monoclonal antibody (gift of G. Gorbsky, University of Oklahoma, Oklahoma City, Oklahoma). Note the presence of the 3F3/2 epitope at centrosomes. (G and H) *polo*^{10} cells showing 3F3/2 staining on the separated kinetochores, and absent from the spindle poles. Bar, 5 μm.

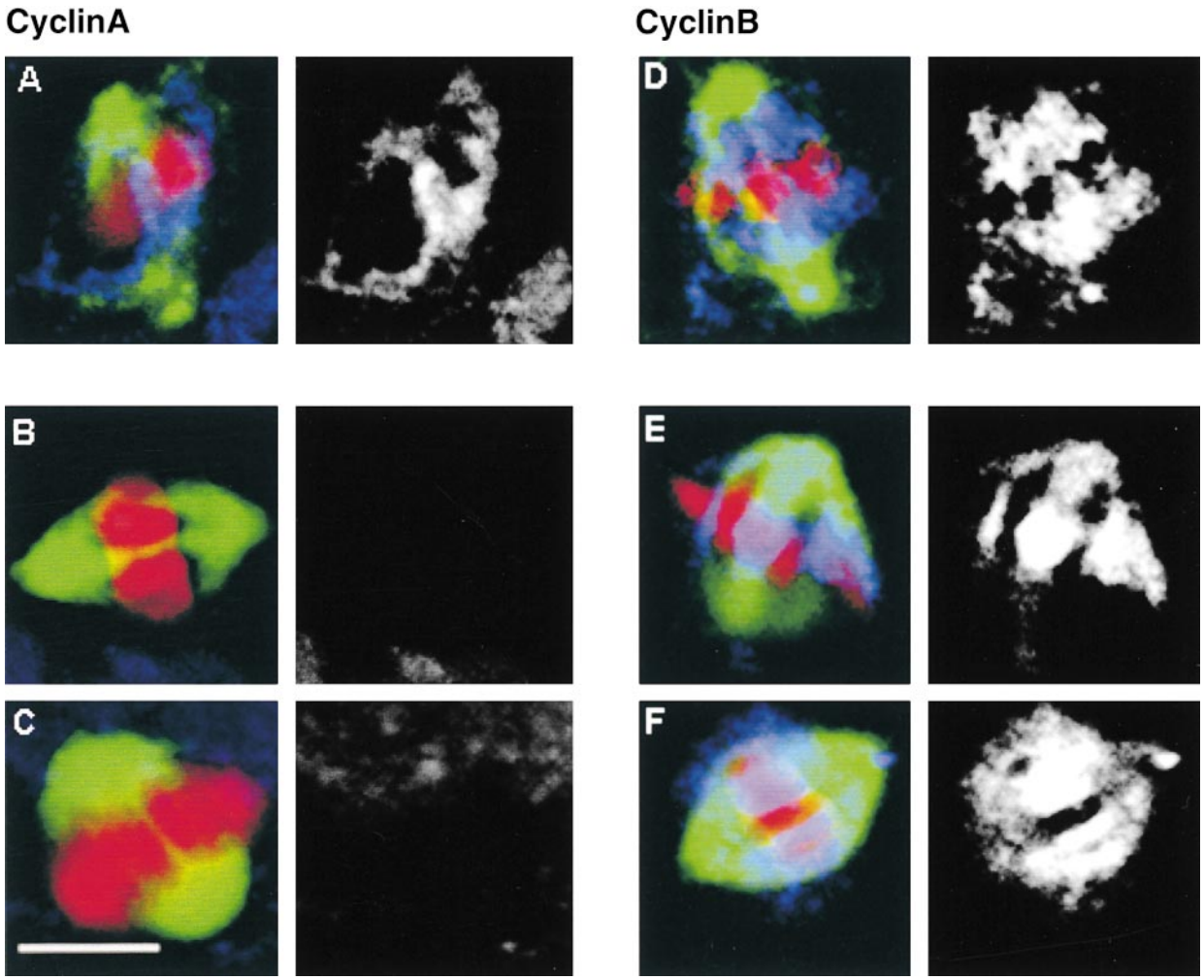

Figure 6. Cyclins A and B in wild-type and *polo* mutant cells. In all panels, spindle microtubules are green, DNA red, and cyclin blue in the merged images. The monochromatic image is of cyclin staining alone using either the antibody Rb270 to detect cyclin A or Rb271 to detect cyclin B (Whitfield et al., 1990). (A) Cyclin A in a wild-type cell at prometaphase. (B and C) Absence of cyclin A staining in *polo*^{9} and *polo*^{10} mutant cells respectively. (D) Cyclin B staining in a wild-type cell at metaphase. (E and F) Cyclin B staining of *polo*^{9} and *polo*^{10} cells, respectively. Bar, 5 μm.

be explained either by a complete failure of chromosome segregation or cytokinesis.

Thus, it appears that although sister kinetochores are under sufficient tension in *polo*^{9} or *polo*^{10} cells to pull them apart by up to 5 μm, the cells are delayed in a metaphase-like checkpoint arrest that can be suppressed by *bub1*.

Discussion

The Recruitment of Centrosomal Antigens

Previous studies on the *Drosophila polo*^{1} allele have revealed requirements for its protein kinase for the function of microtubule organizing centres at several stages of development, and in both chromosome segregation and cytokinesis in male meiosis (Sunkel and Glover, 1988; Herrmann et al., 1998; Riparbelli et al., 2000). The strongly hypomorphic mutant alleles we now describe, *polo*^{9} or *polo*^{10}, suggest a new requirement for Polo kinase in the metaphase–anaphase transition, but also give additional insight into the requirements for Polo kinase to organize microtubule nucleating centers. It is known that both the γ-tubulin ring complex (γ-TuRC) and Asp are required for the integrity of mitotic microtubule organizing centers (Moritz et al., 1998; Schnackenberg et al., 1998; Avides and Glover, 1999), and yet neither appears to be required for localization of the other at the spindle pole. γ-Tubulin is found at the spindle poles in *asp* mutants (Avides and Glover, 1999), and Asp, which only associates with spindle poles during mitosis (Saunders et al., 1997; Avides and Glover, 1999), still localizes to either the poles or the unfocused minus ends of microtubules in mutants of the *dd4* gene that encodes the 91-kD component of the γ-TuRC (Barbosa et al., 2000). The effect of the *polo*$^{9/10}$ mutations on the constitution of centrosomes for the limited number of antigens that we have studied is in fact remarkably similar to that seen when the γ-TuRC is disrupted in *dd4* mutants. In *dd4* cells, γ-tubulin is dispersed throughout the cytoplasm and a large proportion of the CP190 antigen shows a punctate distribution around the condensed chromosomes in much the same way as in *polo*$^{9/10}$ mutants. More-

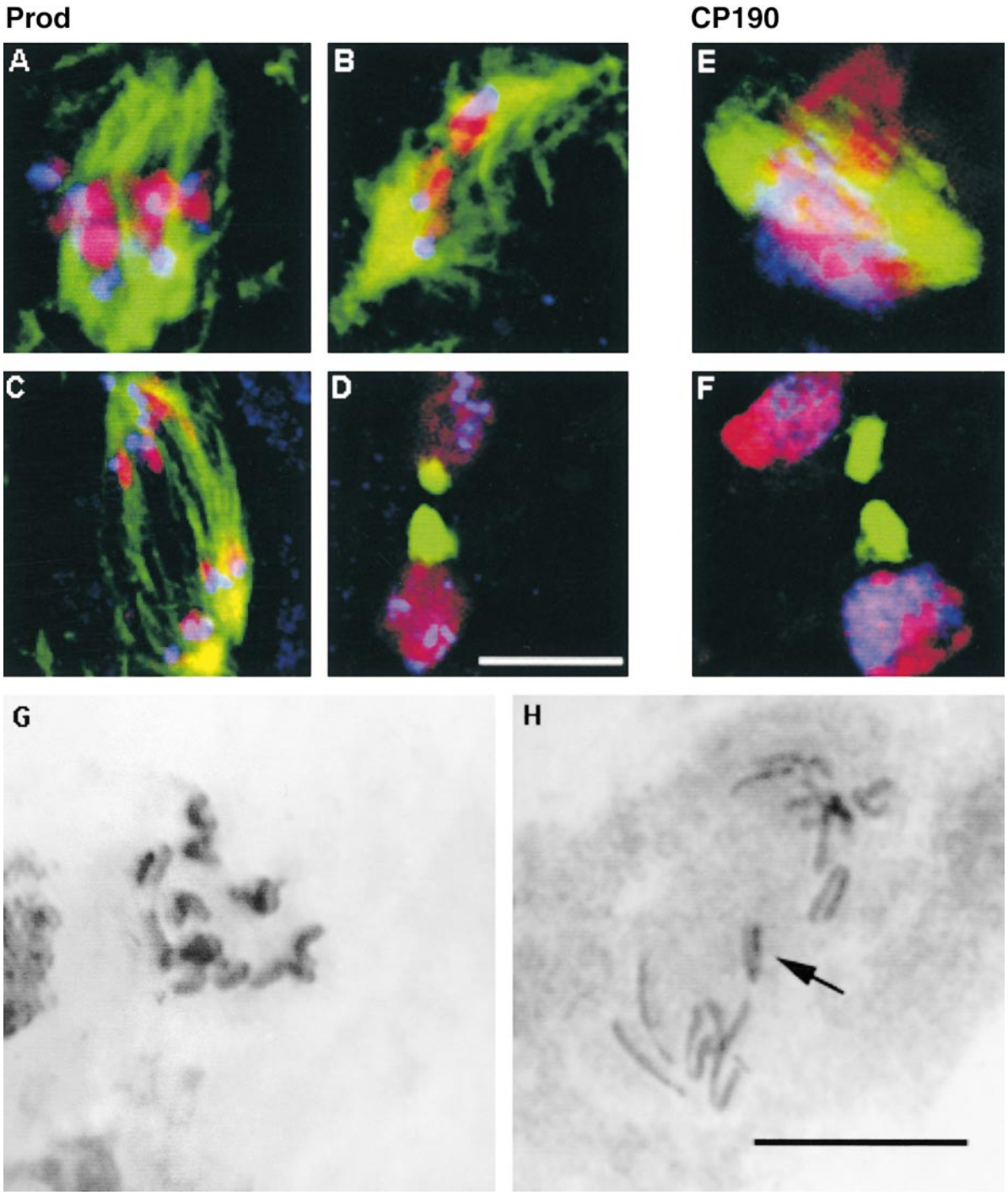

Figure 7. Suppression of the *polo*^{10} mitotic phenotype by *bub1*. In A–F, microtubules are stained green and DNA stained red. (A–D) *polo*^{10} *bub1*^{k6109} cells at metaphase, early anaphase, late anaphase, and telophase, respectively, showing the localization of Prod (blue). (E and F) *polo*^{10} *bub1*^{k6109} cells at early anaphase and telophase showing the localization of CP190 (blue). Bar, 5 μm. (G and H) Giemsa-stained squashed preparations of *polo*^{10} *bub1*^{k6109} cells showing, respectively, sister chromatid separation at metaphase and a lagging chromatid (arrow) at anaphase.

over, in addition to Asp, Centrosomin is present at the focused spindle poles both in *dd4* and *polo*$^{9/10}$ mutants. We suggest that these common aspects of phenotype result from a common primary failure to correctly assemble and/or localize the γ-TuRC at the centrosome. This would be consistent with the observation that γ-tubulin is recruited to the centrosome at mitosis (Khodjakov and Rieder, 1999) and the finding that centrosomes fail to grow and effectively nucleate microtubules in human cells injected with antibodies to Polo kinase (Lane and Nigg, 1996). However, it is of interest that although centrosomes appear to lack γ-tubulin and do not nucleate astral microtubules, they are nevertheless capable of assembling a spindle and of establishing a metaphase-like array of chromosomes.

Metaphase or Anaphase Arrest in polo Cells?

If plks play an essential role in the activation of cdk1, an arrest at the G2/M transition in *polo*^{9} and *polo*^{10} might have been expected. As this is not observed, it suggests either that this function is not required at this stage of *Drosophila* development, or it is redundant, or it can be fulfilled by the little amount of detectable Polo protein. The phenotype of *polo*$^{9/10}$ is more typical of cells unable to pass beyond the metaphase–anaphase checkpoint, as they have low levels of cyclin A and high levels of cyclin B. It is generally believed that the inhibitory signal of this checkpoint arises from unaligned chromosomes and requires a complex containing the Bub1 and 3 and Mad1, 2, and 3 proteins that associates with unattached kinetochores. Mad3 binds to and inactivates Cdc20p-fizzy, a protein that directs the APC to a specific set of substrates. In yeast, one of the Cdc20-APC substrates is Pds1, whose proteolysis releases the separin Esp1 that in turn catalyses the degradation of Scc1p resulting in sister chromatid separation (for a review, see Nasmyth et al., 2000). In the presence of the Bub1 complex of checkpoint proteins, the APC cannot be activated and cells become arrested in metaphase (for a review, see Gardner and Burke, 2000). *polo*$^{9/10}$ cells have Bub1 and the 3F3 epitope at their kinetochores, suggesting they are held in a state of checkpoint arrest by the Bub1 complex. This is confirmed by our observation that *bub1* mutation suppresses the mitotic phenotype of *polo*^{10} and so releases the metaphase-like arrest.

However, *polo*$^{9/10}$ cells exhibit a paradox that sets them apart from other metaphase arrest mutants of *Drosophila*. Their chromosomes appear to have undergone alignment and anaphase movements activated in that sister kinetochores appear to be not only attached through microtubules to the poles but also pulled apart by about 2–5 μm. This itself might have been expected to relieve the checkpoint arrest, and usually the 3F3 epitope is lost from chromosomes under tension. In contrast, Giemsa-stained squashed preparations reveal that only ~17% of sister chromatids remain separated in squashed preparations of *polo*$^{9/10}$ brain cells made in the presence or absence of colchicine. This suggests that the majority of sister chromatids are still held together, explaining their failure to move to the spindle poles. In addition, some chromosomes appear connected in chains through telomeric linkages. Chaining of chromosomes in this way has been described previously in the mutants for the UbcD1 gene that encodes a class I ubiquitin-conjugating (E2) enzyme (Cenci et al., 1997). This observation suggests that Polo kinase may in part play a role in regulating the proteolytic system responsible for breaking such linkages.

Centromere separation seen in intact *polo*$^{9/10}$ cells must be accounted for by pole-directed forces of motor proteins on the chromatids. Although several explanations are possible to resolve the paradox of the *polo* phenotype, the simplest is that Polo kinase is actively required to relieve the function of the Bub1 checkpoint complex in the progression through mitosis. This would have some parallels with the finding that in budding yeast, *CDC5* is essential to escape the metaphase arrest imposed by DNA damage surveillance mechanisms (Toczyski et al., 1997). This hypothesis implies that the *polo* arrest point lies on the cusp of the metaphase–anaphase transition. This is at a later stage than the requirement for the *fizzy* gene product function. In *fizzy* mutants, neither A- nor B-type cyclins are degraded and as we show here, there is no separation of centromeric regions as seen in *polo* mitotic arrest. This incidentally contrasts with mutants for *CDC20,* the *fizzy* homologue of budding yeast where centromeric regions are observed to be separated (Tanaka et al., 2000). However, the transient separation of sister centromeres while chromosome arms still show cohesion does appear to reflect a general aspect of normal progression through metaphase in wild-type budding yeast (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). If similar events occur in metazoan cells, this aspect of normal progression through metaphase might be accentuated in *polo* cells that appear to have initiated anaphase by the criterion of the apparent poleward tension imposed at the centromeres, and yet are not released from "checkpoint" arrest.

Mitotic Exit

Polo kinase may also be required to promote complete separation of sister chromatids and for those specific aspects of APC activity that mediate cyclin B degradation. The latter would have some similarities to budding yeast where mutants in the plk gene *CDC5* are reported to show no effect on Cdc20-APC function and so permit Pds1p to be degraded, in the absence of degradation of the mitotic cyclin Clb2p (Shirayama et al., 1998). Moreover, overexpression of Cdc5p results in proteolysis of Clb2p but not Pds1p, suggesting that the *CDC5* plk promotes Hct1 (Schwab et al., 1997; Visintin et al., 1997; Charles et al., 1998) (*fizzy related*)/APC functions to regulate cyclin B levels. In fact, the phenotype of *polo*$^{9/10}$ mutants resembles that of mutants expressing nondegradable cyclin B in several respects. Expression of stable cyclin B (lacking its destruction box) from a GAL4 responsive promoter in *Drosophila* larval neuroblasts results in a mitotic arrest in which the greater proportion of cells arrest in anaphase (Rimmington et al., 1994). In embryonic cells, the phenotypes resulting from expression of stable forms of cyclins A, B1, and B3 support a sequential requirement for the three proteins: stable cyclin A leads to a metaphase delay, stable cyclin B an early anaphase arrest, and stable cyclin B3 a late anaphase arrest (Sigrist et al., 1995). Sister chromatids clearly separate in those cells expressing stable cyclin B as indicated by in situ hybridization with a dodecasatellite probe to identify the centromeric region of the third chromosomes. A role for *polo* in promoting those aspects of APC activity that mediate cyclin B degradation would be consistent with the observations that mammalian Plk can phosphorylate and activate the APC in vitro (Kotani et al., 1998). However, the ability of the *polo*^{10} *bub1* double mutant to progress through mitosis would suggest that this APC function can also be activated at some level in the absence of *polo* function.

In budding yeast, there is now considerable evidence that Hct1p can be activated by the Cdc14p protein phosphatase (Alexandru et al., 1999; Fraschini et al., 1999; Gardner and Burke, 2000). Cdc14p appears itself to be activated downstream of the GTP-bound active form of Tem1p. This pathway is held in check in response to spin-

dle damage by a two component GTPase activating protein (GAP) formed between Bub2p and the Bfa1/Byr4 protein that promotes formation of the GDP-bound state of Tem1p which does not favor Cdc14 activation. The *CDC5* polo-like kinase appears to regulate the phosphorylation of Bfa1/Byr4 in this process (Lee et al., 2001). In fission yeast, the equivalent pathway regulates the onset of the septation process and appears to be under the control of Plo1 kinase (Tanaka et al., 2001). It is of considerable interest to know the extent to which the pathway might be conserved and whether an analogous process regulates the onset of cytokinesis in metazoans. The requirement for *polo* for cytokinesis in *Drosophila* is obscured by earlier mitotic defects seen in somatic cells with all alleles. The exception is male meiosis, where it seems that spindle checkpoint pathways do not lead to metaphase arrest. However, cells from the *polo*^{9} *bub1* double mutant do attain high levels of polyploidy, suggestive of a failure in cytokinesis once the metaphase arrest is bypassed. It will be a future challenge to determine the extent to which this mitotic exit network has been conserved in metazoans and its relationship to the regulation of the onset of anaphase.

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